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REGULAR ARTICLE

A novel variant of *Oct3/4* gene in mouse embryonic stem cells

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Abstract *OCT4* is a highly conserved gene and plays an important role during early embryonic development and differentiation. Similar to human *OCT4*, mouse *Oct4* gene generates variants. *Oct4A* is a master regulator of self-renewal in pluripotent stem cells. In this study, we have identified a novel Oct4 spliced variant, designated mouse Oct4B, encoding 3 isoforms, termed Oct4B-247aa, Oct4B-190aa and Oct4B-164aa. Furthermore, we have examined the expression pattern of these isoforms in non-pluripotent cells and their function in somatic cell reprogramming. The results revealed the isoforms 247aa, 164aa localized mainly in nucleus and 190aa expressed dotted in the cytoplasm. In contrast to *Oct4A*, *Oct4B* does not function in somatic reprogramming as that of *Oct4A*. Taken together, our data for first time described the intact coding sequence of mouse *Oct4B* and its function in somatic cell reprogramming. These findings will be important for further analysis of the epigenetic mechanisms of reprogramming and highlight the necessity of discriminating Oct4 isoforms in future stem cell research.

Introduction

The transcription factor OCT4 (official symbol POU5F1, also known as OCT3, OCT3/4, OTF3, and OTF4) is an important member of OCT protein family, which is well known for its specific POU DNA binding domain and its diverse function (Scholer, 1991). The human *OCT4* gene encodes three distinct transcripts due to alternative splicing: OCT4A, OCT4B and OCT4B1, these spliced variants share the same domains but have distinct N termini. OCT4A is highly expressed in the nucleus of compacted embryos and blastocyst, and functions as a main regulator in maintaining the pluripotency and self-renewal capacities of ES (embryonic stem) and EG (embryonic germ) cells (Nichols et al., 1998; Okamoto et al., 1990; Pesce et al., 1998; Rosner et al., 1990); Abnormal expression of OCT4A will induce the differentiation of ES cells into endodermal, mesodermal or trophectoderm cells (Matin et al., 2004; Niwa et al., 2000); OCT4A alone or in combination with other factors, is able to reprogram the somatic cells into the progenitor state, indicating an important role of OCT4A in the reprogramming process (Cho et al., 2010; Takahashi and Yamanaka, 2006; Zhao et al., 2009; Zhou et al., 2009); Recently, some groups have published that

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reactivation of OCT4A expression is postulated to occur in differentiated cells that have undergone tumorigenesis (Gazouli et al., 2011; Karoubi et al., 2010).

Compared with OCT4A, human OCT4B encodes a diverse N terminal domain (NTD), localized mainly in the cytoplasm of various non-pluripotent cells and somatic cells (Atlasi et al., 2008). OCT4B cannot sustain ES cell self-renewal, over expression of OCT4B cannot activate transcription from OCT4-dependent promoters, although OCT4A did as reported previously (Lee et al., 2006). In addition, Gao *et al.*, have described that OCT4B mRNA, containing an internal ribosome entry site (IRES), could be alternatively translated into at least three protein isoforms: OCT4B-265aa, OCT4B-190aa and OCT4B-164aa (Gao et al., 2010; Wang et al., 2009).

OCT4B1 is a newly discovered OCT4 spliced variant, which is widely expressed in human pluripotent and non-pluripotent cells. Like OCT4A, OCT4B1 is downregulated in differentiated cells, suggested that OCT4B1 isoform is a putative marker for stemness (Gao et al., 2010; Papamichos et al., 2009).

In mice, *Oct4* encodes at least 2 variants, designated Oct4A and putative Oct4B. Oct4A was detected in somatic stem cells and has been recognized as a gatekeeper for maintaining pluripotency in embryonic stem cells and pre-implantation embryos. However, because of the existence of pseudogene and misinterpretation of background signals, the intact open read frame (ORF) and detailed functions of Oct4B variant remain unclear (Farashahi Yazd et al., 2011; Mizuno and Kosaka, 2008).

In this study, we identified and characterized a novel mouse *Oct4* transcript variant from ES cells, designated as mouse *Oct4B*, whose products share high similarity with human OCT4B. We demonstrated that the mouse *Oct4B* can be translated into three distinct isoforms through endogenous IRES element and alternative initiators of the coding sequence (CDS). In addition, we have found transcript *Oct4B* was not able to reprogram somatic cells to induced pluripotent stem cells, suggesting it was not involved in maintaining the pluripotency of stem cells.

Results

Identification and Cloning of Mouse *Oct4B*

As shown in supplementary Fig. 1, more than two Oct4 protein bands were detected in mouse pluripotent cells. Of note, the 40 kDa protein band represents Oct4A, but other protein bands (putative Oct4B) were unknown. In an attempt to identify Oct4B, mouse genome and EST database were searched for valuable sequence, and based on informative analysis, we amplified a putative *Oct4B* sequence from ES cell cDNA database. It was composed of 741 base pair, encoding 247 amino acids (supplementary Tab. 2 and 3), and was highly similar to human OCT4B (87% identities, Fig. 4B). After transfection with *Oct4B* transcript, three different protein products were detected, with molecular mass of approximately 30 kDa, 23 kDa and 20 kDa (Fig. 1B). The 30 kDa protein was the product of *Oct4B* full-length CDS (247aa), whereas the other two bands needed further validation.

Identification of the Putative IRES Element in *Oct4B* mRNA

Generally, it is known that a single mature mRNA can be translated into a unique protein product, however, with the help of endogenous internal ribosome entry site (IRES), some mRNAs are capable of producing more than two isoforms from the same transcript (Gao, et al., 2010; Wang et al., 2009). Due to Oct4B generated three isoforms (30 kDa, 23 kDa and 20 kDa), therefore we supposed that the coding sequence (CDS) of Oct4B contain internal ribosome entry site (IRES) element. In order to test this hypothesis, bicistronic plasmid RFP-pQCXIN(IRES)-GFP was utilized. As schematic structure shown in Fig. 2A, RFP-pQCXIN(IRES)-GFP is characterized by the existence of two tandem ORFs of RFP and GFP, which is linked by candidate sequence. Generally, the green fluorescent protein could not be translated unless the inserted sequence contains IRES element. In this study, the IRES sequence of the bicistronic vector was replaced with target sequence (1-171nt of *Oct4B*) of the mouse *Oct4B* CDS, followed by transfection into NIH3T3 cells. The results showed (Fig. 2B) after nt 1–117 was introduced in plasmid, GFP expression was observed. Thus this 117-bp fragment at position 1–117 exhibited the IRES activity. Therefore, a putative IRES element was identified in mouse *Oct4B* mRNA.

Identification of the Oct4B Translation Initiation Codons by Site-Directed Mutagenesis

IRES-mediated translation can be initiated in an internal region of the mRNA, resulting in alternative initiation of translation and protein isoforms formation (Macejak and Sarnow, 1991). In eukaryotes, ATG is the dominant initiation codon, but there exist other codons which can initiate translation (Arnaud et al., 1999; Nanbru et al., 1997; Vagner et al., 1995). Because of the existence of additional isoforms and IRES element, we hypothesized that mouse *Oct4B* could use different initiation codons in a single mRNA to encode three protein isoforms. To find out these alternative initiation codons, potential site-directed mutagenesis were induced and followed by identification of their protein products. The results revealed that mutation of CTG (Leucine) to CTT (Leucine) at position 172–174 exclusively abolished the synthesis of 23 kDa product of Oct4B, this result demonstrated that the CTG at 172–174 encoding an isoform of Oct4B containing 190 amino acids; and direct mutagenesis of 250–252 ATG (Methionine) to ATT (Isoleucine) abolished the synthesis of the 20 kDa protein band but not the 30 and 23 kDa protein bands (Figs. 2C, D), thus this result supported that the 20 kDa isoform was synthesized at 250-252nt ATG codon and it contained 164 amino acids. In conclusion, a single *Oct4B* mRNA could encode three isoforms: Oct4B-247aa (AUG-initiated isoform encoding 247 amino acids), Oct4B-190aa (CTG-initiated isoform encoding 190 amino acids), and Oct4B-164aa (AUG-initiated isoform encoding 164 amino acids).

Subcellular Localization of Oct4B Isoforms

Most alternative initiation of translation regulates the subcellular localization of protein isoforms and the sub-location of protein reflects its corresponding functions

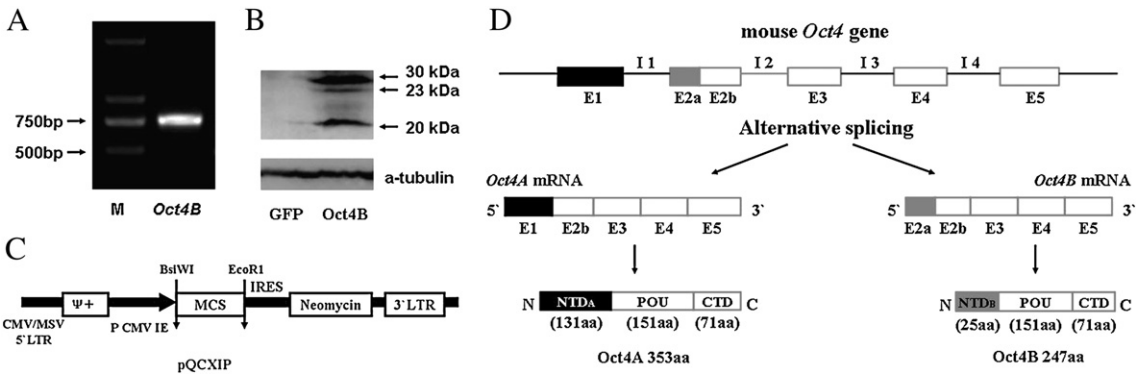

Figure 1 Identification of mouse *Oct4B* transcript. **(A):** The amplification product of *Oct4B* coding sequence (CDS). **M:** DNA marker DL2000. **(B):** Protein expression of *Oct4B*. The CDS of *Oct4B* was inserted into pQCXIP plasmid and transfected into 293 T cells, Western Blot was used to check the protein products. Results revealed *Oct4B* transcript translates three isoforms: 30 kDa, 23 kDa and 20 kDa; pQCXIP-GFP was transfected as negative control. **(C):** The structure of the pQCXIP plasmid. **(D):** Schematic structure of mouse *Oct4* transcripts. The alternative splicing boxes are shown in black and grey colors.

(Land and Rouault, 1998). As we shown mouse *Oct4B* encodes 3 isoforms (Oct4B247aa, Oct4B190aa and Oct4B164aa), though with different length of N-termini, they share the same POU domain, C-termini domain (CTD) and nuclear localization signal (RKRKR) in the domain (Fig. 4A). To investigate the localization of different Oct4 isoforms, we constructed variant expression plasmids fused with GFP protein (Fig. 3A), and Live Cell Image System was used to monitor the distribution of these isoforms. The results turned out that although three isoforms diffuse both in the nucleus and cytoplasm, isoforms Oct4B-247aa and Oct4B-164aa mainly distributed in the nucleus, whereas Oct4B-190aa expressed dotted in the cytoplasm (Fig. 3B), suggesting Oct4B-

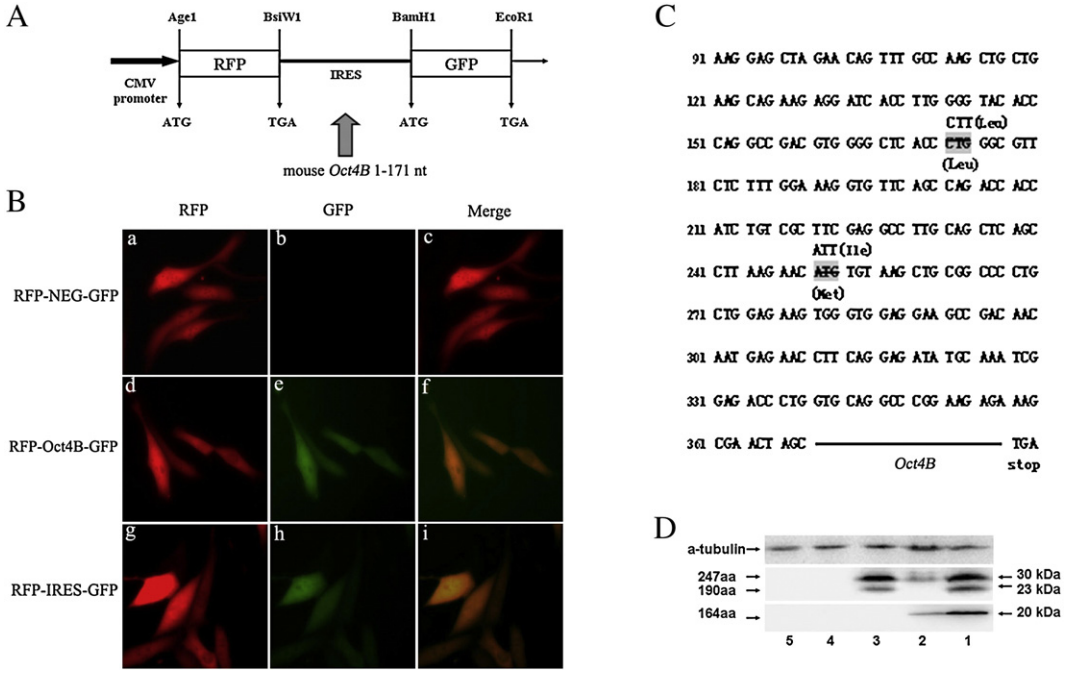

Figure 2 Identification of a putative IRES element in mouse *Oct4B* CDS. **(A):** Schematic structure of RFP-pQCXIN(IRES)-GFP bicistronic plasmid. **(B):** NIH3T3 cells were transfected with bicistronic plasmids, RFP and GFP expression were watched under the Live Cell Imaging System with 490 nm and 537 nm wavelength respectively. The IRES element of bicistronic vector was replaced with 1–171 nt of *Oct4B* CDS. An 80nt random sequence was utilized as the negative control. **(C):** Partial sequence of *Oct4B* CDS. The putative initiation sites were shown: position 172–174 CTG (Leucine) was mutated to CTT (Leucine); 250–252 ATG (Methionine) was mutated to ATT (Isoleucine). **(D):** Western Blot analysis of site-directed mutagenesis. 293 T cells were transfected with mutated vectors: pQCXIP-172-174 and pQCXIP-250-252. pQCXIP-GFP was transfected as negative control. Lane 1: pQCXIP-Oct4B, without any mutation. Lane 2: mutation of CTG to CTT at 172–174 site. Lane 3: mutation of ATG to ATT at 250–252 site. Lane 4: pQCXIP-GFP. Lane 5: 293 T cells.

190aa may function in a different way from Oct4B-247aa and Oct4B-164aa.

The Effects of *Oct4B* on the Reprogramming of Somatic Cells

Recent discoveries in the field of stem cell biology have led to the ability to reprogram somatic cells to pluripotent state in mammals, these cells are termed induced pluripotent stem cells (iPSCs), which have the identical developmental potential as ES cells. This new approach allows the derivation of patient-specific cell lines from individuals with specific diseases (Birgitt Schule., 2011), therefore, it can provide great potential benefits for regenerative medicine (Takahashi and Yamanaka, 2006). As we known, Oct4A is the key factor in the process of iPSC induction. The mouse Oct4B protein shares the same functional POU and CTD domain with Oct4A, with the exception of a diverse NTD. To study the role of Oct4B in the process of somatic cell reprogramming, we introduced *Oct4B*, *Sox2*, *Klf4* and *c-Myc* into mouse embryonic fibroblast (MEFs) by retroviral transduction. After 14 days of consecutive culture, the infected cells were fixed and stained with alkaline phosphatase (AP staining). However, instead of becoming ES-like clones, infected cells died gradually with the extension of culture time and no positive clones emerged from the *Oct4B* infected group, whereas in the positive control group (infected with *Oct4A*, *Sox2*, *Klf4* and *c-Myc*), more than 105 AP staining positive clones were counted (Fig. 4C, supplementary Fig. 2 and 3). These results suggest that Oct4B is not a functional factor in the process of reprogramming as *Oct4A* do.

Of note, many IRES-containing mRNAs encode proteins that play important roles in development, cell cycle progression, cell apoptosis and stress response (Prats and Prats, 2002; Stoneley et al., 2000). Mouse *Oct4B* has IRES element and encodes variants. To investigate whether the expression level of Oct4B would be altered under unfavorable conditions, ES cells (R1) were cultured in hyperpyrexia and hyperxia conditions respectively. Results revealed that under stress conditions, Oct4B-190aa expression was detected upregulated gradually over time (Fig. 5), so it seemed isoform Oct4B-190aa was involved in stress response and maybe act as an anti-stresses protein for the existence of endogenous IRES element.

Discussion

Mouse embryonic stem (mES) cells are an immensely powerful tool for studying the early differentiation of mammalian cell lineages and regenerative medicine. Oct4A is the key regulator in maintaining ES cells pluripotency and self-renewal. However, because of pseudogene expression and alternative splicing, mouse Oct4 variants are still a controversial and unsolved issue (Farashahi Yazd, et al., 2011; Mizuno and Kosaka, 2008). In this study, we clearly demonstrated the existence of *Oct4B* transcript in ES cells and found that Oct4A and Oct4B have different function in reprogramming of somatic cell to iPSC and self-renewal, therefore, it is necessary to distinguish *Oct4A* and *Oct4B* variants in future research.

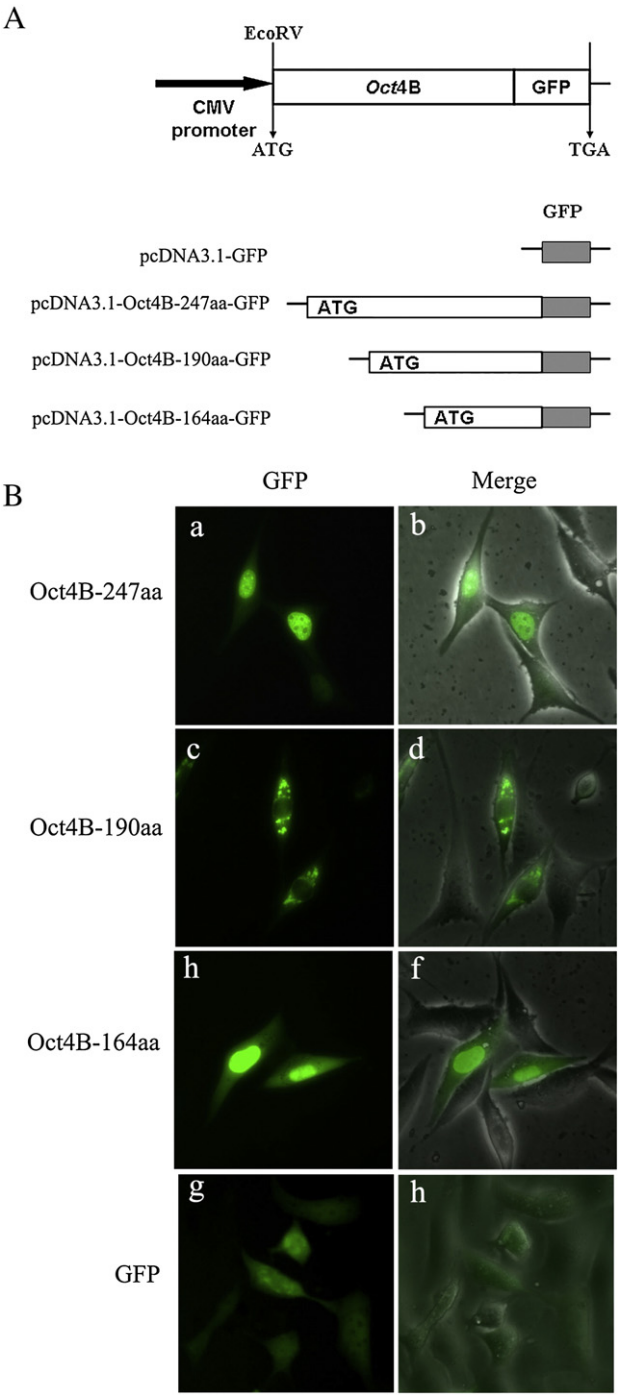

Figure 3 Distribution of mouse Oct4B isoforms. **(A):** Schematic structure of expression plasmid pcDNA3.1-GFP. Isoforms of Oct4B-247aa, 190aa, 164aa were each fused with GFP and transfected into NIH3T3 cells. After 28 hours, fluorescence expression was observed by Live Cell Imaging System under 490 nm wavelength and photos were taken. **(B):** The subcellular distribution of Oct4B-247aa, Oct4B-190aa, and Oct4B-164aa. GFP was used as the control.

First of all, we identified the coding sequence of *Oct4B* transcript, which contained 741 base pairs and encoded 247 amino acids (supplementary Tab.2 and 3). The mouse *Oct4A* gene is known to encode 353 amino acids and encompass 5

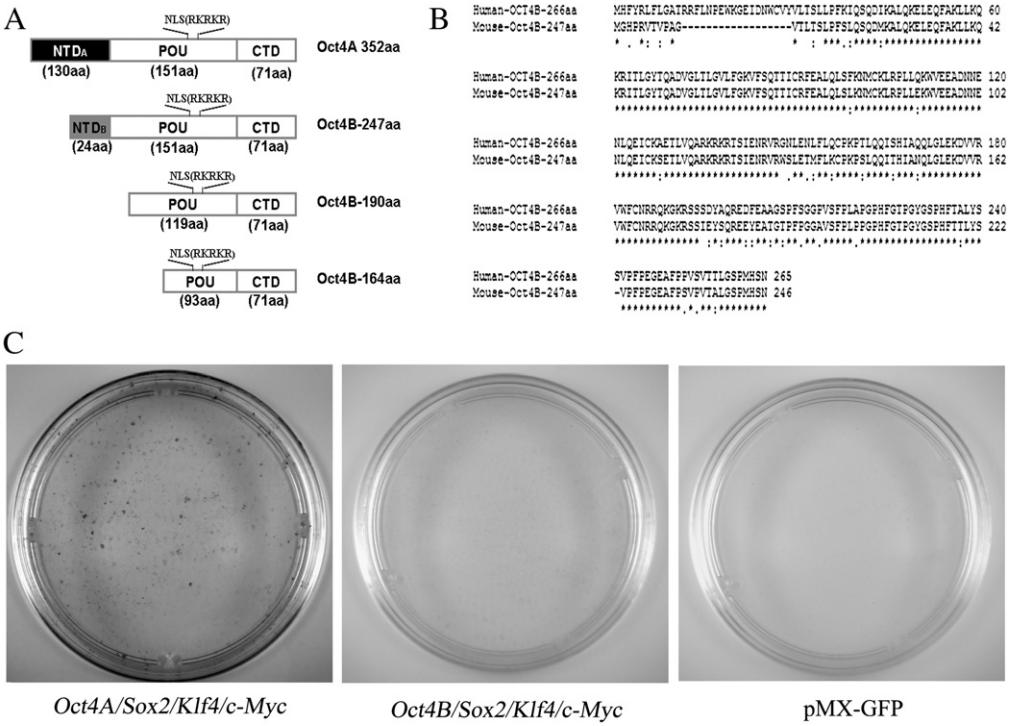

Figure 4 The features and functions of Oct4B. **(A):** Schematic representation of mouse Oct4 isoforms. **(B):** The identities of human OCT4B and mouse Oct4B proteins. **(C):** The alkaline phosphatase (AP) staining of infected mouse fibroblasts after 14 days of consecutive culture. Fibroblasts were infected with two groups of retrovirus: *Oct4A*, *Sox2*, *Klf4*, *c-Myc* and *Oct4B*, *Sox2*, *Klf4*, *c-Myc*, pMX-GFP was infected as negative control.

exons, whereas mouse *Oct4B* is composed of 4 exons: exon 1 contains exon 2 and 66nt of intron 1 of *Oct4A*, exon 2, 3 and 4 are exactly the same with exon 3, 4 and 5 of *Oct4A* CDS (Fig. 1D). Due to the similar nucleic acid sequence between *Oct4A* and *Oct4B*, therefore, primers should be designed in distinctive regions when using PCR analysis: to detect the

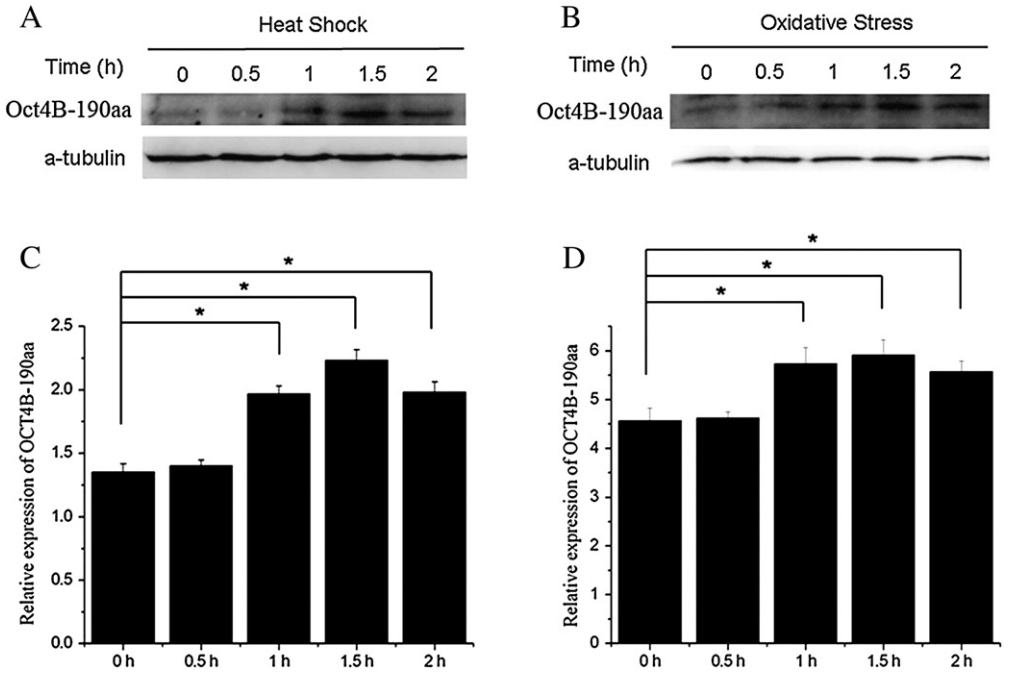

Figure 5 The expression level of Oct4B-190aa under stress conditions. ES cells (R1) were treated with either 42°C (A, C) or 800 uM H_2O_2 (B, D) with different time course, Western Blot analysis was used the check the results. **(C):** The relative expression of Oct4B-190aa under heat shock treatment. **(D):** The relative expression of Oct4B-190aa under oxidative stress treatment.

expression level of *Oct4A*, primers should be set in the exon 1 of *Oct4A*, whereas the 66 bp at 5` end of *Oct4B* should be included when detecting *Oct4B* transcript.

Similar to human *OCT4B*, the mouse *Oct4B* transcript generates three isoforms: Oct4B-247aa (full-length), Oct4B-190aa, and Oct4B-164aa. The protein sequences of these isoforms are highly identical between mouse and human. Oct4B-247aa is the longest product, it's synthesized started from the first ATG initiation site. Compared with the mouse Oct4A, Oct4B-247aa consists of an identical POU domain and C-termini and with different NTD (Fig. 4A). Although Oct4B-190aa shares the same mRNA with Oct4B-247aa, the translation was initiated by another start codon CUG (172–174 position) under the mediation of IRES (1-171nt) element. Oct4B-164aa is the shortest isoform, and the translation was initiated by ATG (250–252 position) codon. Compared with Oct4A, products Oct4B-190aa and 164aa contain intact C-termini and partial POU domain without NTD, so it seems they are not functional copies of the Oct4 protein. Although these three isoforms are generated from a single *Oct4B* mRNA, the endogenous protein isoforms may not be simultaneously expressed in the same cell type, the expression of each isoform may depend on its function and the cell state (Mizuno and Kosaka, 2008). And due to the absence of distinctive primer sequence, Western Blot but not PCR should be the optimal method to distinguish these isoforms.

Combined with Sox2, Klf4 and c-Myc, Oct4B was not able to reprogram somatic cell to iPSC as that of Oct4A, although it contains an identical POU domain and CTD to Oct4A, and this may attributed to the difference of NTD. Human OCT4A protein contains three domains: the N terminal domain (NTD, 1-133aa), encompassing a transcriptional activation region that is active in various cultured cell types; the POU DNA-binding domain (134aa–289aa) that binds to DNA in a sequence-specific fashion, which is essential for its proper function in ES cells (Niwa et al., 2002); and the C terminal domain (CTD, 290aa-362aa) which controls the transactivation function of OCT4 in a cell type-specific way (Brehm et al., 1997). Yet the activity of these two transactivation domains is not identical in ES cells, the NTD domain has a specific function to transactivate a suite of target genes independently of interaction with partners such as Sox2 or cellular E1A-like factors (Niwa, et al., 2002). OCT4A binds its target sequences by the recognition of POU domain and octamer motif (5`-ATGCAATG-3`), and then transactivates the downstream genes with the assistance of the NTD or CTD domain, thus maintaining pluripotency or initiating cell differentiation (Cauffman et al., 2006; Niwa, et al., 2002; Takeda et al., 1992). Compared with OCT4A, human OCT4B shares the conserved POU and CTD domains, but lacks the transactivation domain of the NTD. Moreover, amino acids 1–20 and 21–40 of the NTD have an inhibitory effect on the binding of OCT4B POU domain and target sequence (Lee, et al., 2006). However, overexpression of OCT4B does not interfere with the transactivation reaction activated by OCT4A, and so OCT4B has no effect on pluripotency (Lee, et al., 2006). The mouse Oct4B NTD is composed of 24 amino acids and has no similarity with that of Oct4A. However, it shares a high identity with the human OCT4B: the peptides 1-11aa and 12-23aa of mouse Oct4B are exactly the same as 1-11aa and 29-40aa of human OCT4B NTD which inhibits the transactivation of the POU domain (Fig. 4B). Based on this similarity, it is likely that the 1-23aa of the mouse Oct4B interrupts the binding of the POU domain with target sequence and thus prevents the activation of downstream signal pathways.

There exist two different known mechanisms of protein synthesis from mRNA template: cap-dependent and cap-independent translation initiation. The latter is driven by IRES element that was found in both viral RNAs and cellular mRNAs (Balvay et al., 2009; Shatsky et al., 2010). IRES element was originally discovered in the picornaviral RNA translation process. Viral RNA folds into specific RNA structure to mimic the ribosome scaffold and then recruits ribosomal subunits and translational factors, subsequently starts the elongation process. With reduction of protein involvement and less steps, cap-independent translation dominates when the canonical cap-dependent pathway is interrupted (Fernandez-Miragall et al., 2009; Jackson, 2005). IRES-mediated translation represents a cellular "backup" pool, leading to cell survival or cell death under unfavorable conditions and it confers a regulation mechanism of selective translation at the posttranscriptional level. And this process makes cell respond quickly to rapid changes (Komar & Hatzoglou, 2005; Kozak, 2005). In concordance with other reports (Cheng et al., 2007; Farashahi Yazd, et al., 2011; Karoubi, et al., 2010; Mizuno and Kosaka, 2008), in this study, we illustrated Oct4B-190aa mainly expressed in cytoplasma, although it had nuclear localization signal in POU domain, and the underlying mechanism still need to be further investigated. Meanwhile Oct4B-190aa expression level was upregulated under heat shock and oxidative conditions, suggesting that it was involved in the stress response activity.

In conclusion, mouse *Oct4* gene, regulated by alternative RNA splicing, endogenous IRES element and alternative translation initiation at the posttranscriptional level, may generates 4 isoforms with different function. All of these characteristics of Oct4 contribute to the complexity of its' study. This work highlights the significance of differentiating the expression pattern and biological functions of Oct4 variants.

Materials and methods

Blast and search of mouse *Oct4B* coding sequence (CDS)

Potential mouse *Oct4B* sequences were searched on NCBI EST database and UCSC database (http://genome.ucsc.edu/). Primers were designed for any candidate EST and amplified by PCR (supplementary Tab. 1).

Cell culture and DNA transfection

Mouse embryonic stem cells (R1) were grown as described previously (Nagy et al., 1993). Briefly, cells were cultured in Dulbecco`s modified Eagle`s medium (DMEM) with 15% Gibco fetus bovine serum (FBS), 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 100U/ml streptomycin/penicillin, and 1000U/ml LIF on mitomycin C treated mouse embryonic fibroblast feeders in humidified atmosphere at 37°C with 5% CO_2. When grown

confluence, ES cells were collected and feeder cells were removed for further researches. 293 T and NIH3T3 cells were cultured in DMEM with 10% FBS and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 36 hours, cells were harvested for further assays. To identify the protein expression and subcellular localization, photos were taken with Leica DMIRE2 Live Cell Imaging System directly at 490 nm and 537 nm wavelength respectively.

Total RNA extraction and RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with RNase-free DNase I (Invitrogen) to remove any DNA contamination. Reverse transcription was carried out with 1 μg total RNA using SuperScript III (Invitrogen). RT-PCR was carried out with primerstar HS DNA polymerase according to the instructions (Takara Company). Products were cloned into T vector and more than 10 positive clones were picked and sequenced.

Plasmids construction

Site-directed mutation PCR were performed using primerstar HS DNA polymerase with optimized primers (supplementary Tab. 1). Products were digested with DpnI enzyme for 1 hour at 37°C and then transformed into JM109. Correct clones were picked through sequencing and subcloned into expression vector pQCXIP (Cat. No. 631514, Clontech Company). To find out the potential IRES sequence, various N terminal lengths of *Oct4B* sequence were amplified and cloned into bicistronic vector RFP-pQCXIN(IRES)-GFP, 293 T cells were transfected to examine the effect. Green fluorescent protein (GFP) was fused with Oct4B and its isoforms to detect their distributions. All of the plasmids were sequenced to verify their structure and open reading frames.

Western blot analysis

Total proteins were extracted by RIPA buffer (50 mM Tris, pH7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40) supplemented with 1 mM PMSF, and Western Blot analysis was performed using anti-oct4 antibodies: N-19 was used to recognize Oct4A, Oct4B, Oct4B-190 (1:1000, sc-8628, Santa Cruz Biotechnology); ab19857 was used to recognize Oct4B-164 (1:1000, Abcam Company). The anti-α-tubulin antibody (1:2000, Sigma St Louis) was used as a normalizing control. The reactive bands were detected by chemiluminescence using western lightening (Thermo Company).

The functions of mouse Oct4B

pMXs retroviral vectors-*Oct4A*, *Sox2*, *Klf4*, *c-Myc*, GFP were purchased from Addgene (http://www.Addgene.org), and the induced pluripotent stem cell (iPS) was done as described previously. Briefly (Takahashi et al., 2007), plasmids (pMXs retroviral vectors-*Oct4A*, *Sox2*, *Klf4*, *c-Myc* and pMX-GFP, per 10ug) were tranfected into 8×10^6 Plat-E packaging cells. 36 hours and 72 hours later retrovirus were collected and filtered through 0.45um cellulose acetate filter respectively. 2×10^5 mouse embryonic fibroblast cells (MEF) were infected for 6 hours supplemented with 5ug/ml polybrene, then the medium was changed to R1 medium for consecutive culture. At the 14th day after infection, cells were fixed with 4% polyoxymethylene and stained with alkaline phosphatase (AP) kit. R1 cells were treated with 42°C and 800uM H_2O_2 separately, proteins were collected at 0.5 hour, 1 hour, 1.5 hours, and 2 hours after treatment, Western Blot was used to observe the expression of Oct4B.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2012.04.004.

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